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# The Analysis of Vascular Endothelial Growth Factor Gene Polymorphisms on Clinical and Histopathology Features of Nasopharyngeal Carcinoma

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**Abstract:** Background: Nasopharyngeal malignancy is the first most common malignancy amongst in ear, nose and throat. Incidence rate was 4.7 per 100.000 or 7.000-8.000 cases per year and most of carcinoma is arising from the epithelial cells. Objective: To analyze the VEGF gene variations at +405 C/G and -460 T/C with VEGFR (Flt-4) and LMP-1 tissues expression of nasopharyngeal carcinoma (NPC). Methods: A cross sectional study was carried out with explorative approached at several teaching hospitals in Makassar by Hasanuddin University Research Centre and Eijkman Institute for Molecular Biology on a one-year period, from July 2006 through August 2007. The analysis covered from 90 samples of blood and 45 samples of nasopharynx tissue, consisting of 45 patients for both of the NPC and without NPC as a control. Genomic DNA was extracted from peripheral blood and analyzed by PCR and direct DNA sequencing method for identifying the location of VEGF gene mutation and immunohistochemical expression of VEGFR (Flt-4) and LMP-1 were performed in 45 NPC biopsy samples with avidin-biotin method. Results: The frequencies of +405 C/G and -460 T/C were about 50%, with higher in +405 C/G (hot spot), mostly genotype variant was heterozygote (CG). Our results confirmed that untranslated and promoter region of VEGF gene were higher polymorphic. GC and CC haplotype at +405 C/G and -460 T/C of VEGF gene more susceptible to NPC compared with CT haplotype but no statistical significant. Conclusion: There were no relationships between genotype distribution and allele frequencies at VEGF gene +405 C/G, -460 T/C and -457 T/C with the NPC risk factors.

**Keywords:** Nasopharyngeal Carcinoma (NPC), Vascular Endothelial Growth Factor (VEGF), Latent Membrane Protein (LMP-1)

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## 1. Introduction

The highest incidence of nasopharyngeal malignancy is in Guang Dong Province, China, which are 40–50 cases per 100.000 populations per year. Then it is followed by, Eskimo, Tunisia, Philippines, Malaysia, Algeria and Indonesia. It is rare in whites, Japan, and India. From nasopharyngeal carcinoma profile data report in Hasanuddin University teaching hospital, Makassar from January 2000 to June 2007, it was reported that nasopharyngeal carcinoma (NPOC) accounts 33% of all malignancy in ear, nose, and throat with highest incidence in 4 and 5 decades age groups, male and female proportion; 2-3:1, and most of the histopathology type is anaplastic carcinoma, with the high tendency of metastasis.<sup>1</sup> NPC is often undetected in the early stage because the variations of symptoms and difficulty of

examining the nasopharynx; therefore, the mortality rate is high, which the patients seek medical care in stage III or IV with 10 years survival rate about 10%.<sup>2, 3, 4</sup>

One of the NPC treatments is radiotherapy, which is the main modality; chemotherapy and resection depend on the stage and each hospital protocol. With the low survival rate, patients with late stadium often treated with chemotherapy adjuvant, concomitant, or neo adjuvant with radiotherapy of sandwich fashion, but the result is not satisfying.<sup>4-9</sup>

One of the risk factor for somatic cell malignancy process is gene mutation. Gene mutation can be caused by viral infection, radiation, and carcinogenic substance. Epstein - Barr virus (EBV) is a Herpesviridae family, with gamma Herpes virus subfamily, genera lymphocryptovirus. EBV could be detected with serology test, cell culture, and DNA technology. There are two types of EBV, type 1 and 2. Both

of this type could be found in most of human oropharynx. EBV type 1 is more often found in industrial countries, and type 2 is more often found in Africa and developing countries.<sup>9, 10, 11</sup>

Latent Membrane Protein (LMP-1) product is a integral membrane protein (onkoprotein), was found in 95.6% of NPC patients.<sup>12</sup> EBV LMP-1 induce COX-2 (cyclooxygenase) expresion from NFkB through CTAR1 and CTAR2 (carboxyl terminal activation region) in nasopharyngeal epithelial cells, induce prostaglandine E<sub>2</sub> expresion and increase the production of vascular endothelial growth factor (VEGF), which have a role in NPC angiogenesis.

Certain polymorphisms have been reported in VEGF genes (+405 C/G and -460 T/C) in lung cancer, that is linked to VEGF protein variations.<sup>13, 14</sup> Knowing the VEGF gene DNA sequences, it is expected to know about the individual clinical manifestation of NPC. There is a meaningful relationship between VEGF gene variations on +405 C/G and -460 T/C alleles in squamous cell cancer<sup>13, 14, 15</sup>; therefore in this study, we want to assess the VEGF gene variations on +405 C/G and -460 T/C alleles with stages, histopathology of NPC with different ethnic groups, and if there is a new genetic variation in the NPC patients in Makassar.

## 2. Methods

This study was a cross-sectional study with explorative approach and case-control by analytic. This study conducted in Teaching hospitals in Makassar, from July 2006 to August 2007. The samples were NPC patients who seek care in the teaching hospitals, and met the inclusion criteria, and were taken using non-random sampling. Amount of the cases were 45 and controls were 45.

Staining procedure was the 5 µm thick paraffin block, placed under pre cleaned white glass with poly L lysine coated, then incubated for one night. After the samples were deparaffinized, it is washed using *Phosphat Buffer Saline* (PBS) and citric buffer, then *blocking* normal serum (*Ultra V Block*) was added. Then, primary anti LMP-1 antibody (Novo Costra) and anti VEGF-R(Flt-4) (Lab. Viscon) were added, and stored in refrigerator for 24 hours. Next, the samples were washed by PBS, and were added secondary antibody (Biotinylated Goat Anti Polyvalent), incubated, were washed using PBS, and were added streptavidin peroxidase, and incubated in room temperature. Next, it is washed again using PBS, were added DAB (Diamino benzidine), and were washed using H<sub>2</sub>O, and stained using hematoxillin-eosin. LMP-1 positive expression is seen as brownish granules in membrane and cytoplasm, and VEGF-R is seen as brownish granules in cell cytoplasm. The expression count was done under binocular microscope with 100-200 times magnification and was choosen randomly and counted in 100-200 cells.

## 3. Interpretation

LMP-1 (semi quantitative)

0 : negative, +1 : weak (<10% expression)

+2 : moderate (10% - 25%) +3 : strong (expression>25%)

VEGF- R (Flt-4) (semi quantitative)

0 : negative, +1 : <25% expression

+2 : 26% - 50% expression +3 : 51% - 75% expression

+4 : >75% expression

VEGF DNA extraction was using Chelex method, VEGF DNA amplication:

Primer :

*Forward* : -460F = 5'- CCT CTT TAG CCA GAG CCG GGG -3'

+405F = 5'- CGA CGG CTT GGG GAG ATT GC-3'

*Reverse* : -460R = 5'- TGG CCT TCT CCC CGC TCC GAC-3'

+405R= 5'- GGG CGG TGT CTG TCT GTC TG-3'

After the PCR process, the samples were visualized under UV light. In the sequencing, PCR products were placed in ABI-prism, and compared with human VEGF gene database. Univariate analysis for categories data with nominal and ordinal scales, were using *Chi square test likelihood*, *Chi square test* Spearman and Odds Ratio (OR). Bivariate analysis was using Spearman correlation.

## 4. Results

In July 2006 to August 2007 period, it has been done, a research for VEGF gene polymorphism in NPC based on genotype distribution and its haplotype on +405 C/G, untranslated part and -460 T/C which is VEGF gene promoter in NPC.

On 90 samples, which was consisted of 45 NPC cases and 45 healthy controls. Immunohistochemistry test was done for evaluating VEGFR and LMP-1 expression in cases only, because the control group was not undergone biopsy evaluation. We did not evaluate RFLP (*Retriction Fragment Length Polymorphism*) because enzyme restriction; BsaH I and BsmFI could not be obtained commercially. So, We did direct sequencing from 3ml Cubiti vein blood sample on all samples.

### 4.1. Sample Characteristics

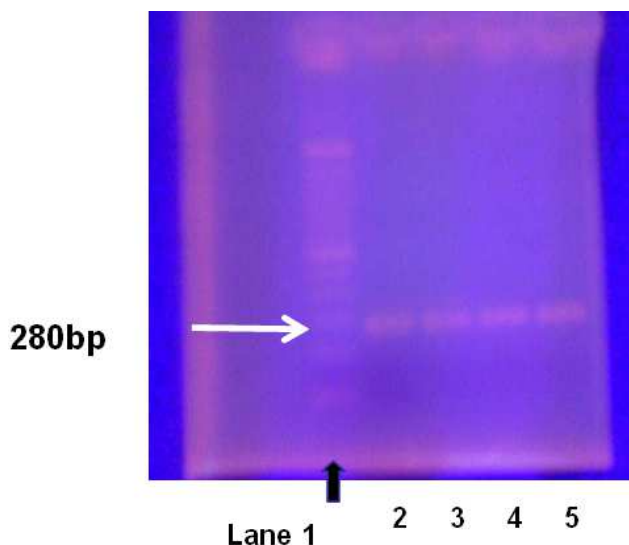
From the sample characteristics, we found the NPC patients age mean is 45.80 ± 13.70 year old. Male are 38 (84.44%) and female were 7 (15.56%). There are two ethnics in our study, Buginese consisted of 21 patients (46.67%) and Makassarese consisted 12 (26.67%). From the control group, the age mean is 39.22 ± 12.53 years.

Primary complaint from NPC patients were bloodstained discharge or epistaxis are 16 (35.56%) followed by 9 cephalgia (20%). The chief complaint was neck mass 24 (53.33%) and nasal obstruction 10 (22.22%). In early stage, we found untypical symptoms, such as unilateral hearing loss, tinnitus, and aural fullness.

### 4.2. PCR Result

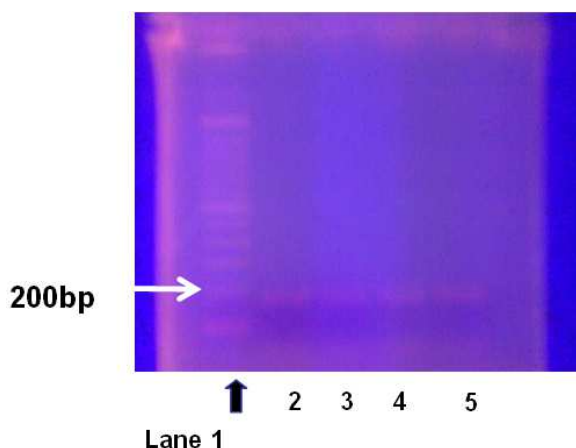
We did PCR test, and succeed amplifying DNA target with

280 bp, with VEGF gene mutation on allele +405 C/G inside (Figure 1.)



**Figure 1.** DNA VEGF allele +405 C/G PCR Product Electrophoresis. Lane 1 : marker 2,3,4 and 5 represents sample with 280 bp.

Then, DNA target amplified with 200 bp, with VEGF gene mutation on allele -460 T/C inside (Figure 2. )



**Figure 2.** DNA VEGF allele -460 T/C PCR Product Electrophoresis. Lane 1 : marker 2,3,4 and 5 represents sample with 280 bp.

#### 4.3. NPC Stage Characteristics

Based on TNM-UICC 2002, we found stage III: 19 (42.22%), next stage IV (IVA, IVB dan IVC): 14 (31.11%) and stage IIB 8 (17.78%).

#### 4.4. NPC Histopathology Characteristics

Based on WHO 1979, we found WHO type II: 15 (33.33%) and WHO type III: 30 (66.67%) and no WHO type I.

#### 4.5. Risk Factor

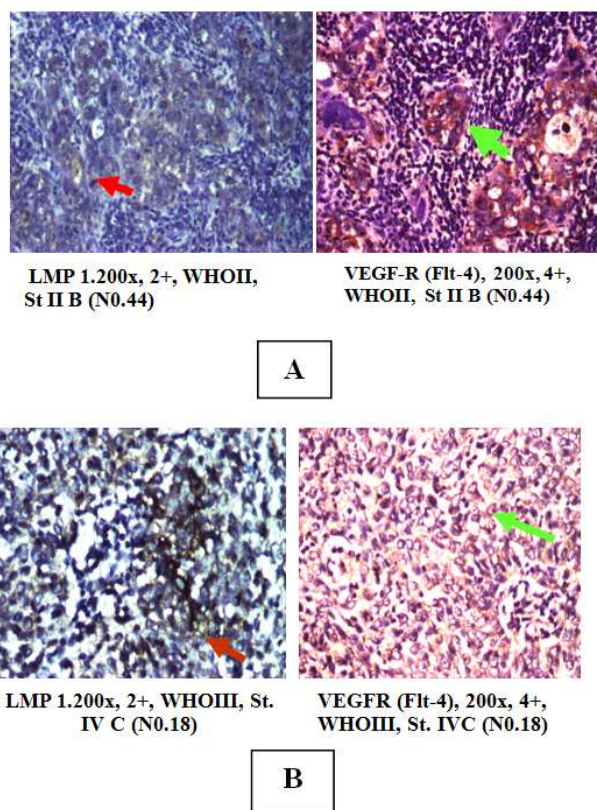
The risk factor in case group was found that the preserved foods, like salted fish, canned food, and smoked meat were up to 95.56%, burned woods and mosquito repellent were up to 82.22%, smoking was 42.22%, alcohol 33.33%, betel

chewere 6.67%, Buginese-Makassarese 32 (71.11%) and others were 13 (28.89%).

The risk factor in control group was found that the preserved foods, like salted fish, canned food, and smoked meat were up to 88.89%, burned woods and mosquito repellent were up to 55.56%, smoking was 51.11%, alcohol 28.89%, betel chewere 11.11%, Buginese-Makassarese 27 (60.00%) and others were 18 (40.00%).

#### 4.6. LMP-1 and VEGF-R (Flt-4) Expression

Immunohistochemistry tests using avidin-biotin method techniques were done in 45 NPC cases biopsy tissues.



**Figure 3.** LMP-1 and VEGF-R (Flt-4) Expression (Immunohistochemistry) WHO Type III and Stage IV C (A). LMP-1 and VEGF-R (Flt-4) Expression (Immunohistochemistry) WHO Type II and Stage II B (B).

Positive expression of LMP-1 in nasopharyngeal biopsy tissue seems like brownish granula in membran and cytoplasm, while in VEGF-R(Flt-4) seems like brownish granula in cell cytoplasm (Figure.3). From primary data, we found LMP-1 expression was 42.1%, and VEGF-R (Flt.4) 100%, there was LMP-1 overexpression ( $\geq +2$ ) as many as 13 (28.89%) dan VEGF-R (Flt-4) 19 (42.22%).

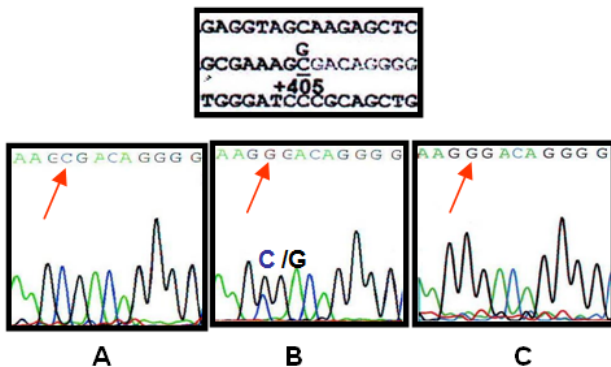
Spearman correlation statistical test for VEGF-R(Flt-4) and LMP-1 expressions role against NPC symptoms, consist of stage based on TNM-UICC 2002 and histopathology characteristic based on WHO 1979, shows no significant correlation between stages and VEGF-R (Flt-4) (0.251) and LMP-1 (0.257) expressions, that means higher VEGF-R (Flt-4) and LMP-1 expression in early stage. There is significant inverted correlation between histopathology characteristic



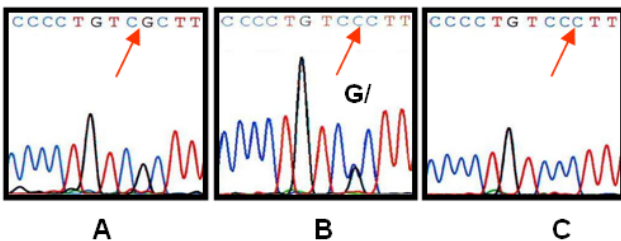
and VEGF-R (Flt-4) ( $r = -0.426$ ,  $p = 0.002$ ) and LMP-1 ( $r = -0.467$ ,  $p = 0.001$ ) expressions, means VEGF-R (Flt-4) and LMP-1 expressions are higher in WHO type II (well differentiated) than WHO type III (poorly differentiated). There is strong correlation ( $r = 0.990$ ,  $p = 0.000$ ) and statistically significant between LMP-1 and VEGF-R (Flt-4) expression. There is no significant correlation (Spearman) ( $p > 0.05$ ) between symptoms, consist of stage and histopathology, LMP-1 and VEGF-R (Flt-4) expressions with alcohol, smoking, betel chewer, pollutants, and preserved foods.

#### 4.7. VEGF Gene Genotype Distribution

CG ( $p = 0.524$ , OR = -) and GG ( $p = 0.467$ , OR = -) Genotype within VEGF gene +405 C/G is not found as risk factor for NPC incidents. While, Allele C (OR : 0.859) against NPC incidents is not statistically significant ( $p = 0.696$ ). Non – CC genotype within VEGF gene +405 C/G is not found as NPC risk factor.

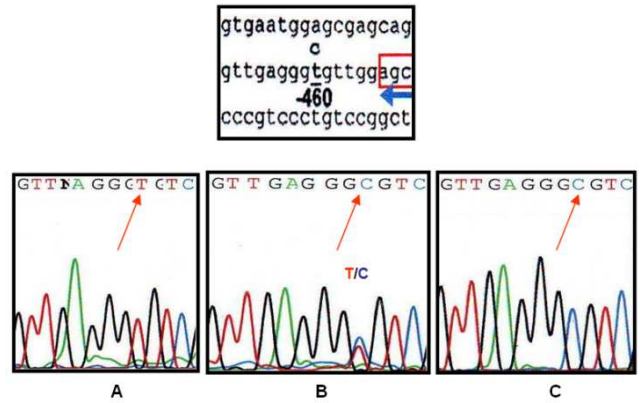


**Figure 4.** DNA Sequencing Electroferogram from VEGF allele +405 C/G with interchanging base from cytosine (C) to guanine (G). A= Wild type, B=Heterozygote and C = homozygote



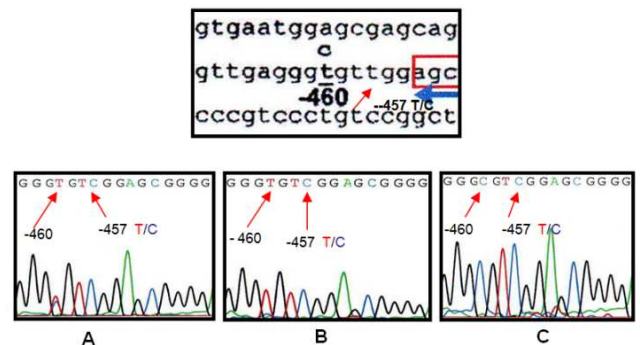
**Figure 5.** DNA Sequencing Electroferogram from VEGF allele +405 C/G (reverse sequence) with interchanging base from cytosine (C) to guanine (G). A= Wild type, B=Heterozygote and C = homozygote

-460 T/C VEGF genes, TC genotype ( $p = 0.548$ , OR = 0.696; 0.213 – 2.276; 95% CI) and CC ( $p = 0.581$ ; OR = 0.648; 0.139-3.036; 95% CI) were not found as risk factor, and so as T allele ( $p = 0.599$ ; OR : 0.809; 0.367-1.785; 95% CI), was not statistically significant and the -460 T/C VEGF gene Non TT genotype was not found as NPC risk factor.



**Figure 6.** DNA Sequencing Electroferogram from VEGF allele -460 T/C with interchanging base from thymine (T) to cytosine (C).. A= Wild type, B=Heterozygote and C = homozygote.

Genetic variation of -457 T/C is a new variant in VEGF gene in NPC, and no one has ever reported it. CC genotype is more than TC genotype, risk estimation could not defined because there was no electroferogram of TT genotype (wild type) .



**Figure 7.** DNA Sequencing Electroferogram from VEGF allele V with interchanging base from thymine (T) to cytosine (C).. A= Wild type, B=Heterozygote and C = homozygote.

#### 4.8. VEGF Gene +405 C/G and -460 T/C Haplotypes

There were no GC, CC, GT, and CT haplotypes distribution difference in cases and controls group. +405 C/G and -460 T/C GC haplotypes have a risk 1.24 times higher to suffer NPC, but it is statistically insignificant. +405 C/G and -460 T/C haplotypes have 3.21 times higher to suffer NPC than CT, but statistically insignificant. Meanwhile, +405 C/G and -460 T/C GT haplotypes could not be proved as risk factor to suffer NPC and statistically insignificant.

#### 4.9. Genotypes Distribution, Allele Frequencies, and VEGF Gene Haplotypes Relationship with Clinical Symptoms (Stages and Histopathology), Expressions (VEGF-R (Flt-4) and LMP-1) and Risk Factors

##### 4.9.1. Mutant Variant with Stages

It could not be proved about genotypes and allele frequencies of VEGF genes +405 C/G ( $p = 0.815$ ,  $p = 0.923$ ), -460 T/C ( $p = 0.548$ -0.599) and -457 T/C ( $p = 0.237$ ,  $p = 0.269$ ) relationship against NPC stages. G allele frequency was

higher than C allele in +405 C/G and C allele was higher than T allele in -457 T/C, while T allele was higher than C allele in -460 T/C against NPC early stages (I-IIA) and late stages (IIB-IVC), but it is statistically insignificant.

#### 4.9.2. Mutant Variant with Histopathology Characteristics

It could not be proved about genotypes and allele frequencies of VEGF genes +405 C/G ( $p=0.854$ ,  $p=0.924$ ), -460 T/C ( $p=0.509$ ,  $p=0.684$ ) and -457 T/C ( $p=0.237$ ,  $p=0.268$ ) relationship against NPC histopathology characteristics. G allele frequency was higher than C allele in +405 C/G, and C allele was higher than T allele in -460 T/C and -457 T/C against histopathology characteristic WHO II, but statistically insignificant. G allele frequency was higher than C allele in +405 C/G in WHO II and III. C allele was higher than T allele in -460 T/C in WHO II, but was lower in WHO III. C allele -457 T/C frequency was higher in WHO II and III, but statistically insignificant.

#### 4.9.3. Mutant Variant with LMP-1 and VEGF-R (Flt-4)

##### Expressions

It could not be proved about genotypes and allele frequencies of VEGF genes +405 C/G ( $p=0.927$ ,  $p=0.969$ ), -460 T/C ( $p=0.532$ ,  $p=0.978$ ) and -457 T/C ( $p=0.579$ ,  $p=0.623$ ) relationship against VEGF-R (Flt-4) expressions in NPC. G allele frequency was higher than C allele in +405 C/G, while C allele was higher than T allele in -457 T/C, and T allele was higher than C allele in -460 T/C and T allele frequency was higher than C allele in -460 T/C against VEGF gene VEGF-R(Flt-4) expression, but statistically insignificant.

It could not be proved about genotypes and allele frequencies of VEGF genes +405 C/G ( $p=0.984$ ,  $p=0.980$ ), -460 T/C ( $p=0.698$ ,  $p=0.893$ ) and -457 T/C ( $p=0.827$ ,  $p=0.839$ ) relationship against VEGF gene LMP-1 expressions in NPC. G allele frequency was higher than C allele in +405 C/G, and C allele was higher than T allele in -457 T/C, and C allele frequency was equal with T allele in -460 T/C against LMP-1 expression, but it is statistically insignificant.

#### 4.9.4. Mutant Variant with Risk Factors

Statistically significant relation could not be proved about genotypes and allele frequencies of VEGF genes +405 C/G and -457 T/C against betel, pollutants, preserved foods, and ethnic group. But there was statistically significant relation between genotype and allele frequency in -460 T/C against alcohol consumption ( $p=0.070$ ) and smoking ( $p=0.041$ ).

Smoking patients with TC genotype in VEGF gene -460 T/C is 4.3 times higher risk to suffer NPC, and statistically significant ( $p=0.034$ ). Smoking habit with CC genotype in VEGF gene -460 T/C is 7.6 times higher risk to suffer NPC, and statistically significant ( $p=0.040$ ). Alcohol drinker with TC genotype in VEGF gene -460 T/C is 5.6 times higher risk to suffer NPC, and statistically significant ( $p=0.025$ ). Alcohol drinker with CC genotype in VEGF gene -460 T/C is 2.6 times higher risk to suffer NPC, and statistically significant ( $p=0.370$ ).

## 5. Discussion

This research was conducted in the form of analyzing VEGF gene polymorphism, including genotypes, allele frequencies, and +405 C/G and -460 T/C haplotypes against clinical symptoms and histopathology in NPC.

From the sample characteristics, Buginese and Makassarese ethnic groups is the most group suffering NPC in Makassar 32 (71.11%), similarly to a study conducted in Makassar in 1997.<sup>3</sup> But it does not represent actual characteristic because Chinese ethnic group was not represented in this study, which is the highest incidence in the world.<sup>16</sup> The age group mean is  $45.80 \pm 13.70$ , means it was in productive age. Male are 38 (84.44%) dan female were 7 (15.56%), with ratio 5 : 1, is higher than the previous study which is 2-3 : 1 and it is probably caused by the smaller samples than before.<sup>1, 6, 12</sup> Primary complaint in NPC patient in this study was epistaxis with 16 patients (35.56%), then cephalgia were 9 patients (20%). The most chief complaint is superolateral neck mass by 24 patients (53.33%), then nasal obstruction with 10 patients (22.22%), and there are untypical symptoms, like tinnitus, aural fullness, unilateral hearing loss in the early stages; different with the past study, there are only two primary complaint, the neck mass and nasal obstruction.<sup>3, 17</sup> It means flu like symptoms with bloodstained discharge and chronic cephalgia, tinnitus or unilateral conductive hearing loss must be suspected as a malignancy until is proven not a malignancy from complete ENT examination. Chief complaints in this study are the same from past study, that are neck mass and nasal obstruction. It means, there is no changing from patients awareness to have an early ENT examination or misdiagnosed in the primary health care.

Based on TNM-UICC 2002 stages, stage III 19 (42.22%) then stage IV (IVA, IVB and IVC) 14 (31.11%) and stage IIB 8 (17.78%), there are no difference from the past study.<sup>2, 3, 4, 17</sup> Based on WHO 1979 histopathology, WHO type III 30 (66.67%), type II 15 (33.33%) and no WHO type I, same as the report that by Fransisca and Perkasa, but it is different from Xie K. that even WHO type III is the most, they found WHO type I in North America up to 25%, in South China is 2%.<sup>17, 18, 19</sup> Khabir et al. reported the WHO type I as many as 1.2 % in North Africa, with WHO type II and III nearly 100%, that WHO type III is 100% correlated with EBV infection.<sup>16, 20, 21</sup> It means, the NPC incidence in Makassar is well correlated with EBV infection. Thus, diagnostic, prognostic, and therapeutic efforts are focused to molecular biology approach against EBV.

In this study, VEGF-R (Flt-4) and LMP-1 expressions from biopsy tissues with immunochemistry test with avidin biotin method technique is found positive expression of LMP-1 in nasopharyngeal biopsy tissue seems like brownish granula in membran and cytoplasm, while in VEGF-R (Flt-4) seems like brownish granula in cell cytoplasm. We found LMP-1 expresion was 42.1%, lower from the study by Khabir A et al. with 60% and VEGF-R (Flt.4) 100%, same with the result by Soo R et al. and higher from Liu XF et al.

that is 61.25%.<sup>15, 22, 23</sup> From LMP-1 overexpression ( $\geq +2$ ) as many as 13 (28.89%) and VEGF-R (Flt-4) are 19 (42.22%), it means not all the NPC express LMP-1, even in EBV infected epithelial cell and actively replicating.<sup>24</sup> Based on that statement, it is needed to find another oncoproteins alternative as a biomarker beside LMP-1, for more accurate diagnostic and prognostic consideration.

VEGF-R (Flt-4) is a receptor from VEGF-C and very important in angiogenesis and lymphangiogenesis, it means there is a regional metastatic potential to lymph nodes is high. This can explain that neck mass is still the chief complaint in NPC patients in Makassar.

Spearman correlation statistical test result for VEGF-R (Flt-4) and LMP-1 expressions role against NPC symptoms, consist of stage based on TNM-UICC 2002 and histopathology characteristic based on WHO 1979, shows no significant correlation ( $p > 0.05$ ) between stages and VEGF-R (Flt-4) (0.257) and LMP-1 (0.251) expressions, that means, higher VEGF-R (Flt-4) and LMP-1 are expressed in early stage. It means VEGF-R (Flt-4) and LMP-1 probably has a role in early stage NPC carcinogenesis, similar to Bcl-2 (Homolog) role in Colorectal carcinoma.<sup>25</sup>

There is no significant correlation between symptoms, consists of stage and histopathology, LMP-1 and VEGF-R (Flt-4) expressions, with alcohol, smoking, betel chewer, pollutants, and preserved foods in NPC. There is a strong correlation between LMP-1 and VEGF-R (Flt-4) expression in NPC, it means the higher LMP-1 expression and VEGF-R (Flt-4) expression is higher too. Thus, LMP-1 is a important regulator (Up-regulator) of VEGF-R (Flt-4) and has a angiogenic potential as a pleiotropic character from LMP-1 beside from transcription activation, gene expression, and cell transformation, cell growth and death.<sup>11, 26</sup>

Sequence variation from VEGF gene could affect VEGF production and activity that causing susceptibility difference between individuals against various of disease like lung carcinoma (squamous cell carcinoma), but from this study in NPC, we found CG, GG and C allele in VEGF gene +460 C/G, it could not prove as a risk factor for NPC incidence in Makassar. Furthermore, new mutant variation in -457 T/C that have not been reported before with TC, CC genotypes and T allele could not be proven as a risk factor for NPC in Makassar. It caused by there was no electroferogram result of TT genotype as a base of wild-type homozygote allele, and complicates the evaluation of genotype distribution and allele frequency.

Non-CC genotype VEGF gene +405 C/G, which in the case of 94.44%, 97.14% controls (OR = 0.971; 95% CI: 0.58 to 16.163) and non-TT genotype -460 T/C in the case of 78.12%, 70.97% controls (OR = 0.684; 95% CI: 0.218 to 2.144) has not been proven as risk factors of NPC in Makassar. The frequency of non CC +405 C/G and non -TT -460 T/C equal those found by Lee S. J. in squamous cell carcinoma of the lung.<sup>13</sup> This means that non-CC and non-TT which is a form of polymorphism +405 C/G and -460 T/C (frequency of more than 1%), as reported by Steven A et al. found as much as 67.50% in breast and endometrial cells in

the UK, Prats AC et al. found 40% of the population of diabetic retinopathy in Japanese, while Lee SJ et al. found in lung squamous cell carcinoma of 55.70% in Korea.<sup>13, 14, 27</sup> Therefore, highly polymorphic VEGF gene especially at positions +405 C/G and -460 T/C as well as the -457 T/C which is a variant of a new mutation that has not been previously reported. It is also no reports of VEGF gene allele polymorphism +405 C/G and -460 T/C on the NPC until we made this study.

Haplotype GC and CC at VEGF gene +405 C/G and -460 T/C at risk of suffering from NPC compared to CT, although it is not statistically significant, while the GT haplotype at VEGF gene +405 C/G and -460 T/C at risk NPC suffer than CT but was not statistically significant. Compared to the results of the studies on breast cells, endometrial and lung squamous cell carcinoma before, is seen a higher frequency of mutations in the variant allele +405 C/G than -460 T/C, as obtained in this study. This means that the allele +405 C/G is a hot spot of VEGF gene. This explains the genetic variants of VEGF are a form of VEGF gene polymorphisms in the promoter region of high.<sup>14</sup>

In the NPC early stage (I - IIA) or advanced (IIB - IVC) turns the G allele frequency was higher than the C allele at +405 C/G, allele C higher than T at -457 T/C and T alleles more higher than the C allele at VEGF gene -460 T/C in NPC patients in Makassar, although it is not statistically significant. So the G allele has a role in the early and advanced stages of NPC, although it is not statistically significant, but still requires further study with a larger sample size.

There were no relationships between genotype distribution and allele frequencies at VEGF gene +405 C/G, -460 T/C and -457 T/C with the NPC risk factors in Makassar, except in person with a variety of TC and CC genotypes VEGF gene -460 T/C with smoking habit 4.3 and 7.6 times likely to suffer from NPC earlier than nonsmokers, and it is statistically significant. Person with TC and CC genotypes variation VEGF gene -460 T/C with alcohol drinking habits of 5.6 and 2.6 times likely to suffer from NPC than non drinking alcohol. From the viewed of genotype TC, CC and allele T at -460 T/C without smoking or alcohol compared to those with the habit of smoking or drinking alcohol on the risk of suffering from NPC, it was obvious the interaction of genetic and environmental on the incidence of NPC. That is phenotypically expression of VEGF gene in the -460 T/C on the NPC is influenced by environmental factors.

Nasopharyngeal carcinoma is still the most common malignancy in head and neck in Indonesia. Buginese and Makassar ethnic groups are still the most group suffering NPC in Makassar with primary complaint and most of the chief complaint is superolateral neck mass, which caused by VEGF-R(Flt-4) overexpression, which is a receptor from VEGF-C, that is an important in angiogenesis and lymphangiogenesis; therefore, has a high potential to regional metastatic of lymphnodes, nasal obstruction, and untypical symptoms, like tinnitus, aural fullness, and unilateral hearing loss in earlier stage, which can be misdiagnosed in the primary healthcare. We found most of them seek

healthcare in stage III, that has lower survival rates and poor prognosis. Means, we need to achieve preventive effort, like health promotion and education for NPC, where smoking and drinking alcohol are still a risk factor for NPC. Furthermore, NPC management needs an intergrated and holistic evaluation with multidiscipline approach that involve public and private society.

Sequence variation from VEGF gene could affect VEGF production and activity that causing susceptibillity difference between individuals against various of disease like lung carcinoma (squamous cell carcinoma), but from this study in NPC, we could not prove it as a risk factor for NPC incidence in Makassar. And, new mutant variation in -457 T/C that have not been reported before with TC, CC genotypes and T allele could not be prove as a risk factor for NPC in Makassar and non-CC genotype VEGF gene +405 C / G, and non-TT genotype -460 T / C has not been prove too as risk factors of NPC in Makassar.

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